

## Evaluation of Repetitive Element Sequence-Based PCR as a Molecular Typing Method for *Clostridium difficile*

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**Repetitive element sequence-based PCR (rep-PCR) is a typing method that enables the generation of DNA fingerprinting that discriminates bacterial strains. In this study, we evaluated the applicability of rep-PCR in typing *Clostridium difficile* clinical isolates. The results obtained by rep-PCR were compared with those obtained by pulsed-field gel electrophoresis (PFGE) and PCR ribotyping. A high correspondence between pattern differentiations produced by rep-PCR and PFGE was observed, whereas PCR ribotyping showed a lower level of discriminatory power.**

Various classes of repeated DNA sequences have been described in diverse prokaryotic genomes (5, 7, 14, 20, 22). Repetitive element sequence-based PCR (rep-PCR) is a new typing method that differentiates microbes by using primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements (16, 25, 26, 28). Multiple amplicons of different sizes can be fractionated by electrophoresis, and the resulting DNA fingerprint patterns, specific for individual bacterial clones, can be compared. Numerous studies have shown that the application of rep-PCR using oligonucleotide primers based on the repetitive extragenic palindromic (REP) elements (REP-PCR) or on the enterobacterial repetitive intergenic consensus (ERIC) sequences (ERIC-PCR) has been successful in typing a variety of bacteria (1, 4, 7, 10, 17, 18, 27).

In the present study, the applicability of rep-PCR in typing *Clostridium difficile*, one of the major causes of hospital-acquired infections (11, 15), was evaluated by using a recent commercial kit that supplied a set of primers complementary to interspersed noncoding repetitive sequences. The results obtained by rep-PCR were compared with those obtained by the reference methods pulsed-field gel electrophoresis (PFGE) and PCR ribotyping (3, 8, 12, 13, 19, 23).

### MATERIALS AND METHODS

***C. difficile* isolates, growth conditions, and DNA extraction.** In performing this study, 34 *C. difficile* clinical isolates, obtained from the collection of the Istituto Superiore di Sanità, Rome, Italy, were chosen. Most of the samples had been previously characterized (21) and were representative of different molecular types. Eight strains were selected from two unrelated outbreaks, 17 were selected from sporadic cases, and 9 were obtained from healthy carriers. All of the strains were toxinogenic except P4, P5, Pd38, Pd27, and Pd32. The isolates were grown on Columbia blood agar (Oxoid) supplemented with 0.1% hemin, 0.1% vitamin K, and 5% yeast extract. The plates were incubated in an anaerobic chamber for 48 h at 37°C.

Purified genomic DNA was extracted using a Nucleobond AXG20 kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions, from

3 ml of overnight brain heart infusion broth (Oxoid) cultures of the different strains.

**PFGE and PCR ribotyping.** PFGE and PCR ribotyping were performed as previously described (21). Briefly, bacterial cultures for PFGE plugs were treated with 4% formaldehyde solution for 1 h to inactivate endogenous nuclease activity (6). The turbidity was adjusted to an optical density of 6.0 at 620 nm. The PFGE plugs were digested with *Sma*I (Boehringer, Mannheim, Germany) as recommended by the manufacturer. PFGE was performed at 6 V/cm for 23 h at 14°C, with an included angle of 120° and linear ramping from 20 to 50 s. PCR ribotyping was performed with primers complementary to conserved regions of the 3' end of the 16S and the 5' end of the 23S rRNA genes. One microliter of purified DNA was used as a template. The PCR assay consisted of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C.

**Rep-PCR.** Rep-PCR analysis was performed using the rep<sub>pro</sub>PCR DNA-fingerprinting kit Uprime-Dt (Bacterial Bar Codes, Inc. Houston, Tex.) kindly provided by Medi Diagnostici s.r.l., Capella Cantone, Italy. Briefly, 1 µl of genomic DNA, at a concentration of 10 to 100 ng/µl, was added to PCR tubes, each containing 24 µl of a PCR mixture composed of 5 µl of PCR buffer (5×), 3.125 µl of deoxynucleoside triphosphates (10 mM each), 0.2 µl of bovine serum albumin (20 mg/ml), 2.5 µl of dimethyl sulfoxide (100%), 1 µl of Uprime-Dt (0.3 µg/µl), and 11.77 µl of high-performance liquid chromatography water. All reagents were supplied in the kit. To each PCR tube, 0.4 µl of rTaq (Takara Shuzo Co., Ltd., Shiga, Japan) was added. After an initial denaturation of 2 min at 95°C, the DNA was amplified for 31 cycles. Each cycle consisted of 3 s at 94°C, 30 s at 92°C, 1 min at 40°C, and 8 min at 65°C. The final extension was for 8 min at 65°C. A positive control, supplied in the kit, and a negative control, consisting of the reaction mixture, were used for each PCR assay. Ten microliters of the PCR products was electrophoresed in a 1.5% agarose gel using 1× Tris-acetate-EDTA buffer mixed with 3.0 µl of ethidium bromide/ml.

**Fingerprint analysis.** The criteria used to analyze the results obtained by the PFGE and PCR ribotyping methods have been previously described (2, 24). The DNA fingerprints of isolates obtained by rep-PCR were compared by visual inspection of the banding patterns. Two isolates were assigned to the same rep-PCR group if their patterns differed by fewer than three bands, according to previously defined criteria (17, 18, 27).

### RESULTS

**Typeability.** PFGE was not able to type all *C. difficile* strains examined. The DNAs of four strains, Px1394, P9, Pd22, and Pd23, appeared consistently degraded during the analysis (Fig. 1). On the other hand, all *C. difficile* isolates could be analyzed by both PCR ribotyping and rep-PCR assay (Table 1 and Fig. 2 and 3).

**Reproducibility.** Reproducibility was carefully evaluated for the 34 *C. difficile* isolates examined by repeating the entire process of typing by each of the three methods two to four times. The profiles obtained by all three techniques were highly

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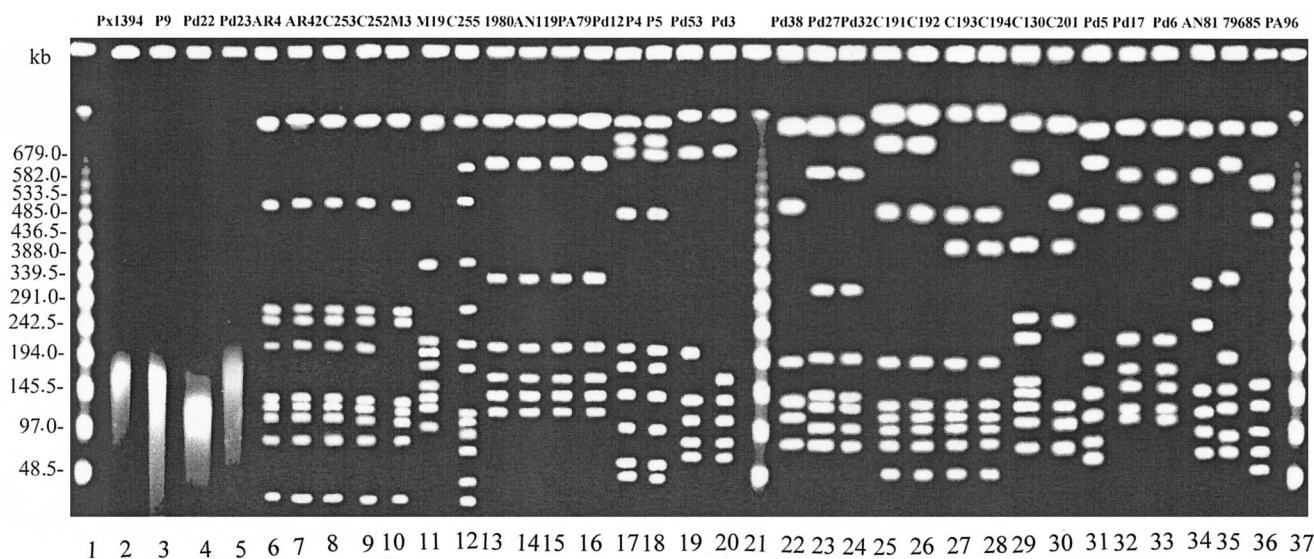


FIG. 1. PFGE of 34 *C. difficile* isolates examined in this study. Lanes: 1,  $\lambda$  ladder PFGE marker; 2 to 5, degraded isolates; 6 to 9, subgroup 1a; 10, subgroup 1b; 11, subgroup 5b; 12, group 7; 13 to 16, subgroup 4f; 17 and 18, subgroup 11a; 19, subgroup 17a; 20, subgroup 17d; 21,  $\lambda$  ladder PFGE marker; 22, group 21; 23 and 24, group 23; 25 and 26, subgroup 27b; 27 and 28, subgroup 27c; 29, group 30; 30, group 31; 31, group 32; 32 and 33, group 33; 34, group 34; 35, group 35; 36, group 36; 37,  $\lambda$  ladder PFGE marker.

consistent with respect to the sizes, numbers, and relative intensities of the fragments obtained for a given isolate.

**Ease of interpretation.** The PFGE restriction profiles comprised 9 to 17 distinct, well-resolved fragments of 48.5 to 679.0 kb and were relatively easy to interpret and compare (Fig. 1). PCR ribotyping generated banding patterns ranging from 0.506 to 1.018 kb. Analyses of the negative images of gels were necessary for easier discrimination of banding patterns (Fig. 2). Rep-PCR yielded 5 to 11 intensely stained fragments that ranged in size from 7.0 to 0.3 kb and were easily identified (Fig. 3).

**Discriminatory powers.** The discriminatory powers of the different techniques were defined by the number of distinct typing groups and subgroups identified among the *C. difficile* isolates examined and the total number of distinct patterns detected (Table 2). Overall, PFGE and rep-PCR gave generally comparable results, while PCR ribotyping identified approximately half as many distinct patterns. Among the 34 isolates examined, PFGE discerned fewer groups and subgroups than rep-PCR (16 versus 19 and 19 versus 22, respectively) because of the nontypeability of four isolates by PFGE.

**Comparison of results obtained by PFGE, rep-PCR, and PCR ribotyping.** A comparison of the results of PFGE, rep-PCR, and PCR ribotyping is shown in Table 1. For the typeable *C. difficile* isolates, these data show that all isolates were grouped by rep-PCR exactly as by PFGE, whereas only 67% of the isolates were grouped similarly by PCR ribotyping. As also observed in a previous study (21), strains belonging to a single PCR ribotype were classified as belonging to different groups by PFGE and rep-PCR.

The four strains not typed by PFGE were divided into three groups (XIII, XVI, and XVIII) using rep-PCR. While PCR ribotyping combined two strains into one group, D, rep-PCR was able to recombine them into two different groups, XIII and XVIII; rep-PCR group XVI corresponded to PCR ribotype group F.

TABLE 1. List of *C. difficile* isolates examined in this study and summary of molecular typing results

Clinical isolate	Source	Group/subgroup identity by:		
		PFGE	rep-PCR	PCR ribotyping
Px1394 <sup>a</sup>	Sporadic case		XVI	F
P9	Sporadic case		XVIII/XVIIIa	D/D1
Pd22	Sporadic case		XVIII/XVIIIb	D/D2
Pd23	Sporadic case		XIII	D/D2
AR4	Outbreak 1	1/1a	I	A
AR42	Outbreak 1	1/1a	I	A
C253	Outbreak 1	1/1a	I	A
C252	Outbreak 1	1/1a	I	A
M3	Carrier	1/1b	I	A
M19	Sporadic case	5/5b	X	C
C255	Sporadic case	7	XIX	C
1980	Carrier	4/4f	V/Va	D/D2
AN119	Carrier	4/4f	V/Vb	D/D2
PA79	Sporadic case	4/4f	V/Va	D/D2
Pd12	Sporadic case	4/4f	V/Va	D/D2
P4	Carrier	11/11a	VI	G
P5	Carrier	11/11a	VI	G
Pd53	Sporadic case	17/17a	VII/VIIa	M
Pd3	Sporadic case	17/17d	VII/VIIb	M
Pd38	Carrier	21	XV	Q
Pd27	Carrier	23	IX	T
Pd32	Carrier	23	IX	T
C191	Outbreak 2	27/27b	II	A
C192	Outbreak 2	2727b	II	A
C193	Outbreak 2	27/27c	II	A
C194	Outbreak 2	27/27c	II	A
C130	Sporadic case	30	III	A
C201	Sporadic case	31	IV	A
Pd5	Sporadic case	32	XVII	R
Pd17	Sporadic case	33	VIII	P
Pd6	Sporadic case	33	VIII	P
AN81	Sporadic case	34	XII	D/D2
79685 <sup>a</sup>	Sporadic case	35	XIV	D/D2
PA96	Carrier	36	XI	T

<sup>a</sup> *C. difficile* isolates Px1394 and 79685 were kindly provided by I. R. Poxtan (Edinburgh, United Kingdom) and T. Karjalainen (Chatenay-Malabry Cedex, France), respectively.

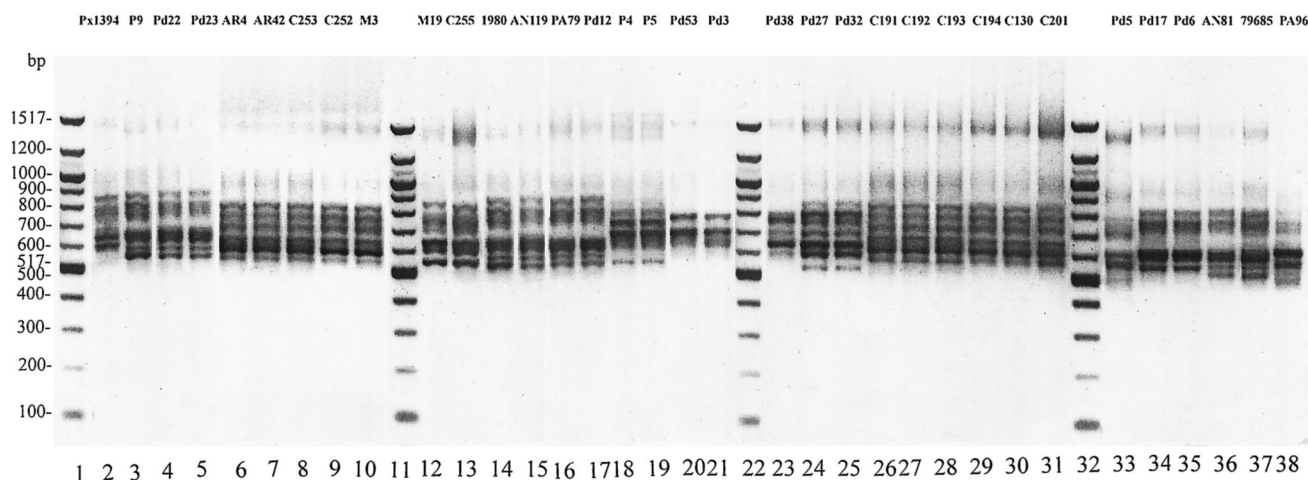


FIG. 2. PCR ribotyping patterns identified among 34 *C. difficile* isolates examined in this study. Lanes: 1,  $\phi$  x174 *Hae*III digest; 2, PCR ribotype F; 3, PCR ribotype D/D1 (group/subgroup); 4 and 5, PCR ribotype D/D2; 6 to 10, PCR ribotype A; 11,  $\phi$  x174 *Hae*III digest; 12 and 13, PCR ribotype C; 14 to 17, PCR ribotype D/D2; 18 and 19, PCR ribotype G; 20 and 21, PCR ribotype M; 22,  $\phi$  x174 *Hae*III digest; 23, PCR ribotype Q; 24 and 25, PCR ribotype T; 26 to 31, PCR ribotype A; 32,  $\phi$  x174 *Hae*III digest; 33, PCR ribotype R; 34 and 35, PCR ribotype P; 36 and 37, PCR ribotype D/D2; 38, PCR ribotype T.

We observed an exact correspondence between PFGE subgroups 17a and 17d and the rep-PCR subgroups VIIa and VIIb, whereas PFGE subgroups 27a and 27b were not discriminated by rep-PCR and were both categorized as pattern II. In contrast, the rep-PCR subgroups Va and Vb were both recognized as PFGE subgroup 4f. Only in the case of *C. difficile* isolates P9 and Pd22 did PCR ribotyping discriminate two subgroups, D1 and D2, corresponding to rep-PCR subgroups XVIIIa and XVIIIb, respectively.

### DISCUSSION

Rep-PCR generates DNA fingerprints that allow the discrimination of bacterial strains (16, 25, 26, 28). The term rep-

PCR refers to the general methodology involving the use of oligonucleotide primers based on short repetitive sequence elements dispersed throughout the bacterial genome. Palindromic units, or REP elements, and ERIC sequences are the most commonly used targets for DNA typing (5, 7, 22). Rep-PCR has been demonstrated to be a useful typing technique for a variety of bacteria (1, 4, 17, 18, 27).

In this study, rep-PCR demonstrated a higher discriminatory power than PCR ribotyping in analyzing both related and unrelated *C. difficile* isolates, and the results obtained by rep-PCR were comparable to those obtained by PFGE. Among isolates from outbreaks, rep-PCR and PCR ribotyping discriminated 2 and 1 groups, respectively, and among unrelated isolates, rep-

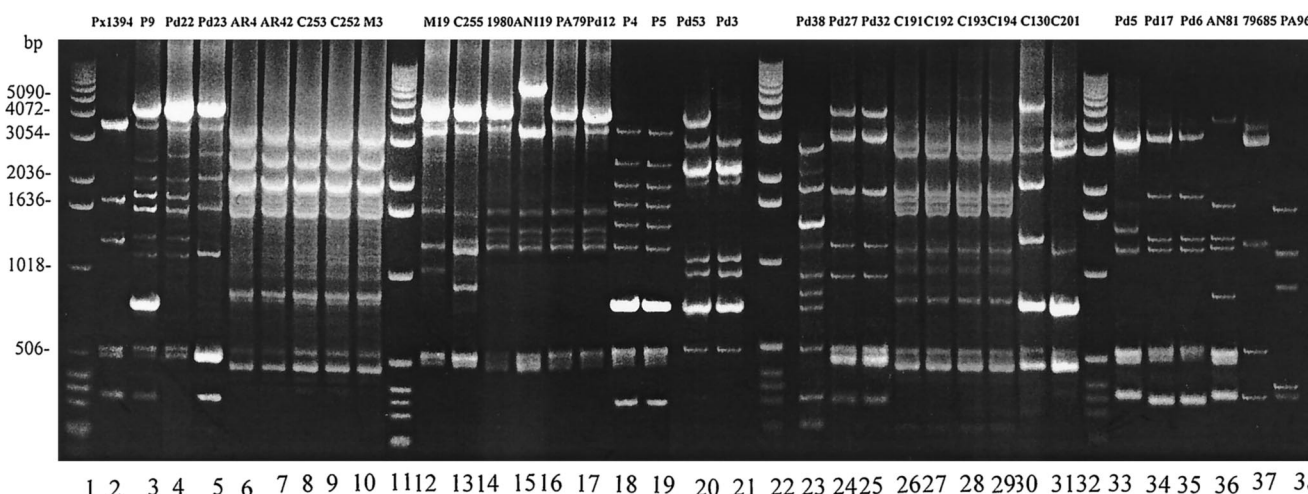


FIG. 3. Rep-PCR fingerprints of 34 *C. difficile* isolates examined in this study generated using the rep<sub>pro</sub>PCR DNA-fingerprinting kit Uprimer Dt. Lanes: 1, 1-kb ladder; 2, type XVI; 3, type XVIII/XVIIIa (group/subgroup); 4, type XVIII/XVIIIb; 5, type XIII; 6 to 10, type I; 11, 1-kb ladder; 12, type X; 13, type XIX; 14, type V/Va; 15, type V/Vb; 16 and 17, type V/Va; 18 and 19, type VI; 20, type VII/VIIa; 21, type VII/VIIb; 22, 1-kb ladder; 23, type XV; 24 and 25, type IX; 26 to 29, type II; 30, type III; 31, type IV; 32, 1-kb ladder; 33, type XVII; 34 and 35, type VIII; 36, type XII; 37, type XIV; 38, type XI.

TABLE 2. Discriminatory powers of different molecular methods for typing *C. difficile* isolates

Clinical source	No. of isolates	No. of groups/no. of subgroups identified by:		
		PFGE	rep-PCR	PCR ribotyping
Outbreak	8	2/3	2/2	1/1
Sporadic cases	17	10/11	14/16	7/8
Carriers	9	6/6	6/7	5/5
Total	34	16/19	19/22	10/11

PCR discriminated 14 groups, whereas PCR ribotyping discriminated only 7. The four isolates that were untypeable by PFGE were recognized as belonging to three groups by rep-PCR and were recognized as belonging to two groups by PCR ribotyping. Because of the nontypeability of these four isolates, rep-PCR was able to distinguish a greater number of distinct patterns overall (22 versus 19) than PFGE, and a complete correspondence between PFGE and rep-PCR subgroups was not demonstrated in this study.

Interspersed noncoding repetitive DNA sequences have been preferentially found in gram-negative bacteria (25, 27). Limited data describe the presence of repetitive DNA elements in clostridia and the applicability of rep-PCR to the genus (9). The high discriminatory power of rep-PCR in *C. difficile* typing, first observed in this study, was related to the numerous bands of the patterns generated, which allowed good differentiation among *C. difficile* isolates. These results indicate that the *C. difficile* genome harbors repetitive element sequences in discrete numbers. In contrast, these kinds of sequences were demonstrated to be rare in the *Clostridium botulinum* genome (9). Further studies of clostridia should be carried out to determine the dissemination of these sequences in the genomes of the different species of the genus.

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